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Bleomycin genotoxicity and amifostine (WR-2721) cell protection in normal leukocytes vs. K562 tumoral cells

Annamaria Buschini[a], Chiara Alessandrini[a], Anna Martino[a], Luca Pasini[a], Vittorio Rizzoli[b], Carmelo Carlo-Stella[c], Paola Poli[a,*], Carlo Rossi[a]

[a]*Istituto di Genetica, Università degli Studi di Parma, Parco Area delle Scienze 11/A, 43100 Parma, Italy*
[b]*Dipartimento di Medicina Interna e Scienze Biomediche, Università di Parma, via Gramsci 14, 43100 Parma, Italy*
[c]*Istituto Nazionale Tumori, via Venezian 1, 20133 Milano, Italy*

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Abstract

Recent advances in chemotherapy have focused on the benefit of high dose regimens, increasing the dose intensity of conventional chemotherapy. However, unacceptable cytotoxicity and genotoxicity on normal cells often impairs the proper management of patients. Phosphoaminothiol WR-1065, the active metabolite of amifostine, appears to protect normal cells and tissues against cytotoxic exposure to radiation or chemotherapeutic agents. Nevertheless, there is disagreement in findings on amifostine protection against bleomycin-induced severe side effects which have suggested that amifostine effectiveness against bleomycin-induced genotoxicity in normal leukocytes and tumour line cells K562 be studied. DNA damage was detected by single cell gel electrophoresis (or Comet) assay, a technique able to detect DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells and which appears to be an ideal tool for assessing variability in response of different cell types *in vitro*. WR-2721 appears to selectively protect healthy leukocytes but not K562 tumoral cells. On the other hand, data on the inter- and intra-individual sensitivity to bleomycin and amifostine suggest that individual metabolic/genetic differences and other factors relating to lifestyle may be responsible for response variability. Application of the Comet assay in appropriate clinical settings to test the sensitivity of patients when undergoing chemotherapy appears possible.

Keywords: Comet assay; Antiblastic drugs; DNA damage; Individual variability; Chemotherapy; Chemoprotection

1. Introduction

A high dose antineoplastic therapy can be associated with severe side effects. Cyto- and genotoxicity induced by chemotherapy can limit the dose and duration of treatment, adversely affect patient quality of life, and may be life-threatening. Therefore, a selective protection of normal vs. tumour tissues by cytoprotective agents seems to be an important goal. Amifostine (WR-2721) is designed to improve or eliminate toxicity to normal cells, without reducing the antitumour efficacy [1,2]. This selective protection mainly derives from the lower levels in tumour tissue of alkaline phosphatase, a membrane-bound enzyme able to dephosphorylate WR-2721 to the active metabolite WR-1065. This active thiol, which is able to scavenge free radicals [3] and conjugate to electrophilic compounds [4], might be expected to reduce the toxicity and clastogenicity in clinical applications of various antiblastic drugs.

The antibiotic bleomycin is a *S*-independent radiomimetic antitumoral agent with unique genotoxic properties [5,6]. The drug is a free radical-based DNA damaging agent which induces a mixture of strand breaks and abasic sites by highly specific, concerted free radical attack on deoxiribose moieties in both DNA strands [5,7–10]. A cellular thiol such as glutathione seems to potentiate the clastogenic action of bleomycin and participate in the formation of toxic metabolites of this drug [11]. On the other hand, increase in glutathione content enhances cell resistance to bleomycin [12–14]. However, there is evidence that in cells, cytochromes rather than glutathione may act as the reductants [8,10].

* Corresponding author. Tel.: +39-0521-905608; fax: +39-0521-905604.
E-mail address: mutgen@unipr.it (P. Poli).

Abbreviations: WBC, white blood cells; PBS, phosphate buffer saline; DMSO, dimethyl sulphoxide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

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In humans, bleomycin can cause significant morbidity and mortality when used to treat malignancies such as lymphoma and testicular carcinoma. Several studies on rodents regarding the protective effect of amifostine against bleomycin-induced injuries showed contrasting findings. In one case, amifostine significantly decreased the amount of acute lung injury and subsequent fibrosis in hamster [15]; the same group [16] confirmed by studies in mice that pre-treatment with amifostine reduces the pulmonary toxicity induced by bleomycin administration. On the other hand, Ortiz *et al.* [17] found that amifostine exacerbated bleomycin-induced lung injury in mice.

The contradictory findings on thiol action against bleomycin-induced DNA damage [11–14] and amifostine protection against bleomycin-induced severe side effects [15–17] suggest further investigations are needed on the combined action of amifostine and bleomycin on normal vs. tumour cells.

The aim of this study was to verify the selective protective effect *in vitro* of amifostine against bleomycin-induced DNA damage in white blood cells (WBC) vs. the acute myelogenous leukaemia cell line K562. K562 cells and normal WBC were utilised to better represent susceptibility to the drug *in vivo*. Our previous findings (unpublished) had shown a complete lack of alkaline phosphatase in the K562 cell line, while its presence is known in normal leukocytes.

After drug treatment, DNA damage was evaluated by alkaline single cell gel electrophoresis assay (SCGE or Comet assay) under condition associated with minimal cytotoxic effects to reduce as much as possible the potential for DNA migration associated with cell death. The Comet assay, a technique allowing DNA damage in a single cell to be shown in an epi-fluorescence microscope [18,19], is able to detect DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells. The Comet assay proves to be an ideal tool for assessing variability in response of different cell types *in vitro* [20–22].

2. Materials and methods

2.1. Chemicals

Amifostine (Ethyol®) was provided by Schering-Plough and Bleomycin (Bleomicina) by Rhône-Poulenc Rorer; styrene oxide and all other laboratory chemicals were purchased from Sigma-Aldrich.

2.2. Cells

EDTA-anticoagulated peripheral blood was obtained by venipuncture from consenting healthy smoker and non-smoker donors. In order to isolate leukocytes, the blood was maintained at 37° for 5 min in an erylysis buffer (155 mM NH_4Cl, 5 mM $KHCO_3$, 0.005 mM Na_2EDTA, pH 7.4), centrifuged and washed with phosphate buffer saline (PBS), and finally resuspended ($\sim 10^6$ cells/mL) in RPMI-1640 medium (Gibco).

The K562 acute myelogenous leukaemia cell line was maintained in the suspension culture in RPMI-1640 supplemented with 10% (v/v) foetal bovine serum (FBS, Stem Cell Technologies) and L-glutamine (2 mM). Exponentially growing K562 cells, washed with PBS and resuspended ($\sim 10^6$ cells/mL) in RPMI-1640 medium, were used throughout this study.

2.3. Bleomycin and amifostine treatment

Appropriate volumes of bleomycin were added to an Eppendorf tube containing cell suspension at a final volume of 1 mL. The cells were treated at 37° with or without WR-2721 (3 mg/mL) 15 min prior to treatment for 1 hr at 37° at different doses of bleomycin, and then washed twice in PBS.

The chosen doses were within the range in which a dose–response relationship could be found that would show minimal cytotoxic effects to avoid false positive results associated with toxicity-induced DNA degradation. Cell viability and apoptotic cells were checked and the Comet assay performed only with a viability $\geq$95% (Trypan blue exclusion method) and apoptotic cells $\leq$4%. The apoptosis percentage was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay [23].

2.4. Alkaline single cell gel electrophoresis assay

The assay was performed basically according to Singh *et al.* [18]. Degreased slides were previously dipped in 1% normal melting agarose for the first layer. The cells ($\sim 2 \times 10^5$ cells) were then mixed with 85 μL of 0.7% low melting agarose (LMA) and placed on the first layer. Lastly, 85 μL of LMA were added as the top layer. The cells were lysed at 4° in the dark overnight in an ice-cold freshly prepared solution of 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulphoxide (DMSO), pH 10. The slides were then placed on a horizontal gel electrophoresis unit. The DNA was allowed to unwind and express alkali-labile sites as single strand breaks for 20 min in an electrophoretic alkaline buffer (1 mM Na_2-EDTA, 300 mM NaOH, pH 13) and subjected to electrophoresis for 20 min at 0.78 V/cm and 300 mA. Alkali and electrophoresis treatments were performed in an ice bath. All the steps already described were performed under a yellow light to prevent additional DNA damage. Once electrophoresis had been carried out, the slides were gently washed in a neutralisation buffer (0.4 M Tris–HCl, pH 7.5) to remove alkali and detergent. Subsequently, the slides were dried and fixed by immersion in absolute ethanol for at least 60 s. The slides were

prepared in duplicate for each sample. Immediately before the examination, the DNA was stained with 100 μL ethidium bromide (10 μg/mL). The samples were examined at 400× magnification under a fluorescent microscope (Leitz Dialux 20) equipped with an excitation filter BP 515–560 nm and a barrier filter LP 580 nm, using an automatic image analysis system (Cometa Release 2,1-Sarin). The image analysis system gives a quantitative description of Comets by various parameters such as migration distance towards anode of DNA fragments, between the edge of the Comet head and end of the tail, head diameter, and percentage DNA fluorescence intensity of total DNA in the head and tail of the Comet. The tail moment is an integrated value considering both the distance and amount of migrated DNA, i.e. tail length × tail % DNA fluorescence intensity. In this system, the tail moment is strictly related to migration distance ($R^2 = 0.98 \pm 0.01$; 10 independent experiments, 10^3 cells per experiment). The DNA percentage shows low variations and weakly affects tail moment values. The Comet parameter Comet length and tail moment were chosen to represent the data on genotoxic effects. One hundred cells (50 cells in two slides) per sample, selected at random, were analysed under constant sensitivity.

The results are presented as frequency distributions of single cell DNA damage or as box- and whisker-plots. In this case measured values at the tested concentrations are shown as boxes that include 50% of the data. The top and bottom of the boxes mark the 25th and 75th centiles and the inner line marks the median value; 25% of data above the 75th centile and 25% below the 25th centile are marked as ''whiskers'' limited by the maximum or minimum values. Outliers are displayed as points. Negative (0.1% DMSO) and positive (50 μM styrene oxide) controls were performed. The samples were coded and evaluated blind. All the tests were generally performed three times.

2.5. Cell survival assay

The cells, pre-treated with 0 or 3 mg/mL of amifostine for 15 min and then treated with various doses of bleomycin as previously described, were washed, resuspended in RPMI-1640 and maintained in a humidified incubator at 37° with 5% CO_2. Cell survival was detected by Trypan blue exclusion, 24 hr after treatment. The tests were performed in triplicate.

2.6. Statistical analysis

The data were analysed using the statistic and graphic functions of SigmaPlot 5.0 and SigmaStat 2.0. A one-way analysis of the variance test was performed. If a significant F value ($P \leq 0.05$) was obtained, the comparison between the grade of DNA damages was analysed by using Dunnett's C-test.

3. Results

Normal WBC and K562 leukemic cells, pre-incubated at 37° with or without WR-2721, were analysed for the increase in electrophoretic mobility of nuclear DNA after exposure to increasing concentrations of bleomycin. A range of drug concentrations (0, 50, 100, and 150 μg/mL) was previously tested for cell toxicity. The chosen doses (0–100 μg/mL) were within the range showing, immediately after treatment, a cell survival ≥95%. The highest concentration (150 μg/mL) showing a higher toxicity (69% in WBC and 62% in K562, respectively) was rejected. Amifostine dose and time pre-treatment were chosen from previous studies [24–26].

A previous analysis of inter-individual variability had been performed in four different donors (Table 1). Smoker

Table 1
Inter-individual variability of DNA damage induced by different doses of bleomycin in WBC of four subjects

Dose (μg/mL)	Amifostine	Subject 1 (smoker)	Subject 2 (non-smoker)	Subject 3 (non-smoker)	Subject 4 (non-smoker)	Average mean (subjects 1–4)	Average mean (subjects 2–4)
		Comet length (μm)					
Untreated		14.67 ± 9.40	14.74 ± 8.89	13.36 ± 1.55	13.29 ± 2.77	14.02 ± 0.80	13.80 ± 0.82
0	−	16.75 ± 6.76	14.14 ± 10.66	12.56 ± 3.52	13.11 ± 3.26	14.14 ± 1.86	13.27 ± 0.80
50	−	27.49 ± 17.93	16.75 ± 13.35	23.32 ± 13.50	18.60 ± 11.09	21.54 ± 4.84	19.56 ± 3.39
100	−	41.49 ± 17.03	28.05 ± 11.37	24.01 ± 15.88	31.21 ± 12.36	31.19 ± 7.47	27.76 ± 3.61
0	+	15.06 ± 8.89	14.56 ± 7.59	13.66 ± 7.08	12.44 ± 3.51	13.93 ± 1.15	13.55 ± 1.06
50	+	18.16 ± 7.84	13.71 ± 6.53	17.08 ± 11.43	14.58 ± 7.64	15.88 ± 2.08	15.12 ± 1.75
100	+	28.45 ± 15.55	20.19 ± 9.15	17.02 ± 9.21	18.73 ± 9.22	21.10 ± 5.07	18.65 ± 1.59
		Tail moment (μm)					
Untreated		11.14 ± 7.14	10.51 ± 6.34	8.68 ± 1.01	8.59 ± 1.79	9.73 ± 1.29	9.26 ± 1.08
0	−	12.96 ± 5.23	10.13 ± 7.64	8.17 ± 2.29	8.72 ± 2.17	9.99 ± 2.14	9.01 ± 1.01
50	−	23.46 ± 15.30	12.79 ± 12.79	19.41 ± 11.24	14.71 ± 8.77	17.59 ± 4.80	15.64 ± 3.41
100	−	36.65 ± 15.04	23.19 ± 9.40	19.66 ± 13.00	27.24 ± 10.79	26.69 ± 7.33	23.36 ± 3.79
0	+	11.93 ± 7.04	10.03 ± 5.23	9.11 ± 4.72	7.94 ± 2.24	9.75 ± 1.68	9.03 ± 1.05
50	+	14.11 ± 6.09	9.40 ± 4.48	13.26 ± 8.87	10.72 ± 5.62	11.87 ± 2.19	11.13 ± 1.96
100	+	24.92 ± 13.62	15.62 ± 7.08	12.90 ± 6.98	14.70 ± 7.24	17.03 ± 5.38	14.41 ± 1.38

donor (subject 1) shows a significant difference with respect to non-smoker subjects (Dunnett's C, $P < 0.001$). Bleomycin shows a greater effectiveness on cells of subject 1. The mean ratio of DNA damage induced by bleomycin with/without amifostine is 0.71 ± 0.07 with a protection of about 30% without significant difference among the subjects. Although the three non-smoker subjects were not significantly different, we performed all the subsequent experiments on cells of the same donor (subject 2, one of the author) to eliminate residual variability.

Three independent experiments were performed on WBC and K562 cells (Figs. 1 and 2). The average mean and the average median values of the Comet length and tail moment are reported together with the average 95th percentiles. The dispersion of the values, i.e. variance/mean, is also shown (Tables 2 and 3).

Styrene oxide (50 μM), a compound which acts directly on DNA [27], was used as a positive control. Styrene oxide strongly induced DNA damage (mean Comet lengths were 43.11 ± 3.68 and 47.38 ± 5.30 μm in WBC and

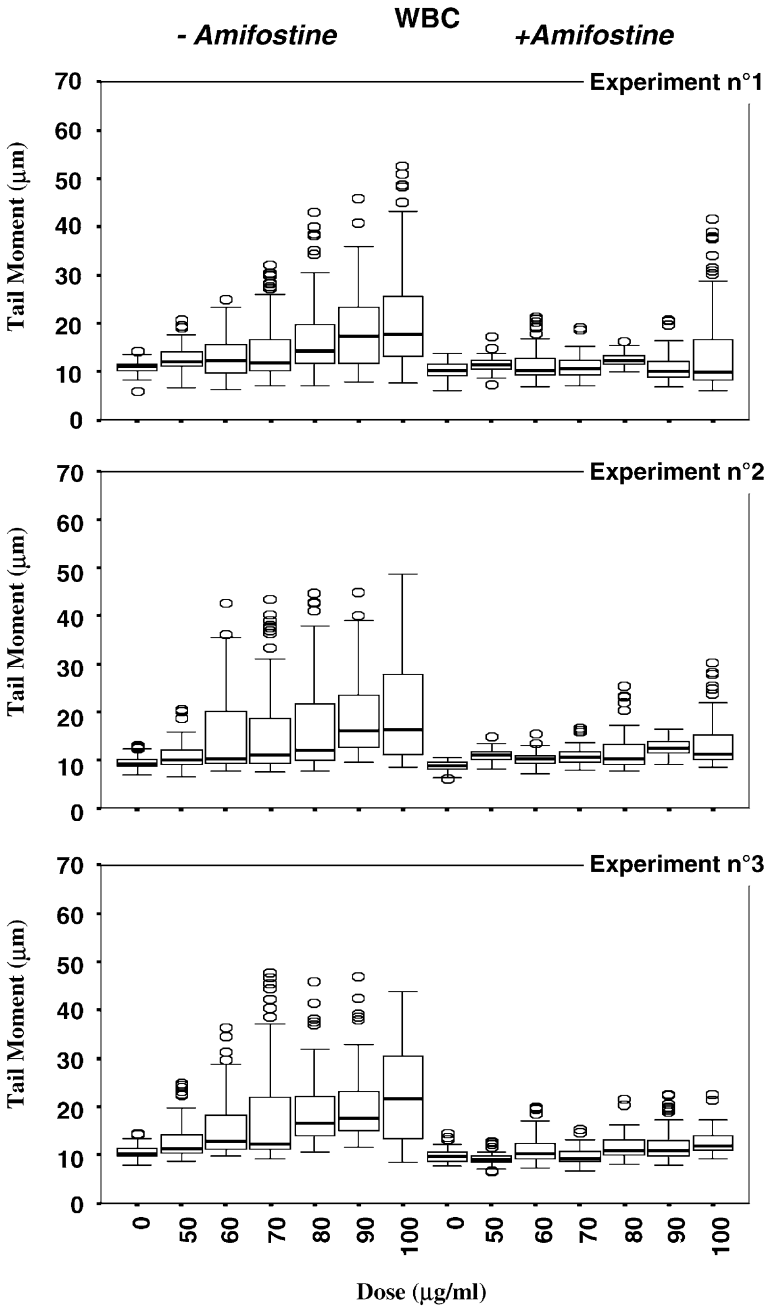

Fig. 1. Genotoxic effects of different doses of bleomycin, with (+) and without (−) amifostine, in WBC: the results of three independent experiments are presented by the Comet parameter tail moment. Results from 100 Comet images per treatment are presented in box and whisker-plots (see Section 2).

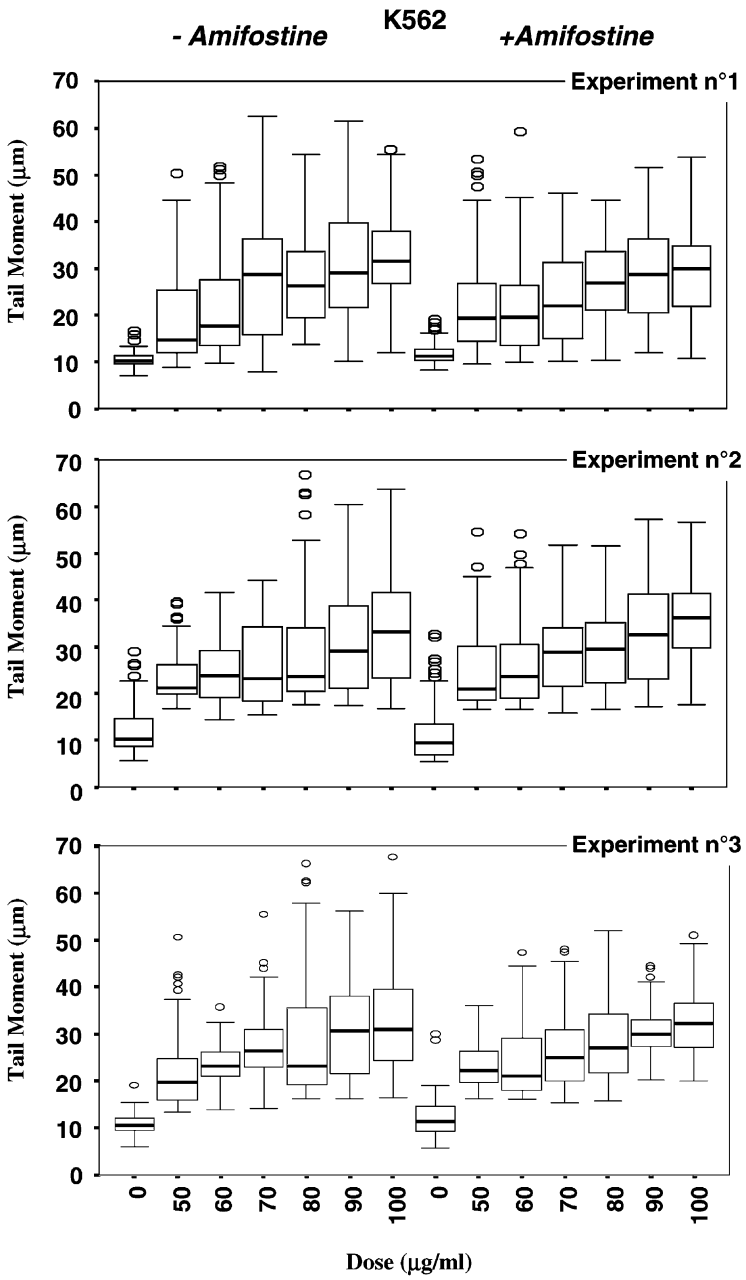

Fig. 2. Genotoxic effects of different concentrations of bleomycin, with (+) and without (−) amifostine, in K562 cells: the results of three independent experiments are presented by the Comet parameter tail moment. Results from 100 Comet images per treatment are presented in box and whisker-plots (see Section 2).

K562, respectively). ''Ghost cells'', in which DNA is extensively degraded allowing most of the Comet head to migrate under electrophoresis, were not observed in both WBC and K562.

Apoptosis data ($\leq$4%), detected by TUNEL assay immediately after treatment, confirm the lack of hedgehog cells, such as cell viability that immediately after treatment is always $\geq$95%.

This high survival percentage does not preclude later appearance of cytotoxicity. Therefore, cell survival was measured after 24 hr (Table 4). In actual fact, the data do not show any clear relationship with drug doses, cellular types, or treatment (w/o amifostine). At the bleomycin used doses, efficient mechanisms can probably repair DNA damage. However, further investigations will have to be performed to clarify these issues.

In WBC, bleomycin increased DNA damage in a linear dose-dependent manner and the protective action of amifostine was shown: pre-treatment with thiol-reduced DNA damage (Table 2).

The mean Comet length and tail moment for WBC treated with bleomycin at 100 μg/mL are doubled as

Table 2
DNA damage induced by different doses of bleomycin with (+) or without (−) amifostine pre-incubation in WBC[a]

Dose (μg/mL)	Amifostine	Average mean (μm ± SD)	Average median (μm ± SD)	Average 95th percentile (μm ± SD)	Dispersion ± SD (variance/mean)
		Comet length			
0	−	14.06 ± 0.89	14.07 ± 1.19	17.45 ± 0.14	0.38 ± 0.15
	+	13.89 ± 0.96	13.68 ± 1.08	16.84 ± 2.42	1.23 ± 0.96
50	−	17.91 ± 1.40	15.33 ± 1.36	35.41 ± 9.04	3.26 ± 1.85
	+	15.26 ± 0.36	14.27 ± 1.76	22.45 ± 11.44	1.54 ± 2.57
60	−	19.55 ± 1.28	16.38 ± 1.96	33.18 ± 5.13	3.13 ± 1.38
	+	15.62 ± 0.18	14.48 ± 0.03	25.21 ± 2.90	1.61 ± 0.95
70	−	20.74 ± 2.30	16.41 ± 0.87	40.93 ± 7.88	4.63 ± 2.34
	+	16.28 ± 0.76	14.40 ± 1.23	35.01 ± 8.6	3.89 ± 1.56
80	−	22.24 ± 2.03	19.17 ± 3.12	44.37 ± 2.20	4.67 ± 1.04
	+	16.99 ± 0.47	15.06 ± 1.41	28.15 ± 9.64	2.47 ± 1.74
90	−	23.66 ± 1.48	21.15 ± 0.78	43.31 ± 4.41	4.11 ± 0.58
	+	18.13 ± 0.90	15.47 ± 1.71	36.03 ± 10.05	4.25 ± 2.72
100	−	26.58 ± 2.08	23.52 ± 3.46	47.40 ± 2.12	5.22 ± 0.69
	+	19.29 ± 1.06	15.30 ± 1.52	37.72 ± 8.01	4.54 ± 2.63
		Tail moment			
0	−	10.38 ± 0.66	10.20 ± 0.86	12.75 ± 0.10	0.33 ± 0.18
	+	9.88 ± 0.68	9.54 ± 0.75	12.39 ± 1.78	1.16 ± 1.06
50	−	13.68 ± 1.07	11.12 ± 0.99	30.44 ± 7.77	3.75 ± 1.51
	+	11.40 ± 0.27	10.54 ± 1.30	18.96 ± 9.66	1.85 ± 2.82
60	−	14.81 ± 0.97	11.81 ± 1.41	28.16 ± 4.33	3.49 ± 1.19
	+	11.32 ± 0.13	10.19 ± 0.02	20.68 ± 2.38	1.43 ± 1.07
70	−	16.06 ± 1.78	11.75 ± 0.62	36.15 ± 6.96	4.87 ± 1.89
	+	12.49 ± 0.58	10.22 ± 0.85	33.81 ± 8.33	4.21 ± 1.93
80	−	17.78 ± 1.62	14.32 ± 2.33	39.06 ± 1.94	4.53 ± 0.81
	+	12.88 ± 0.36	11.14 ± 1.04	23.43 ± 8.02	2.13 ± 1.81
90	−	19.30 ± 1.21	16.82 ± 0.62	38.07 ± 3.88	3.79 ± 0.49
	+	13.86 ± 0.69	11.10 ± 1.23	32.86 ± 9.17	4.57 ± 3.06
100	−	21.29 ± 1.67	18.58 ± 2.73	42.75 ± 1.91	5.01 ± 0.58
	+	14.39 ± 0.79	10.94 ± 1.09	33.98 ± 7.21	4.73 ± 2.56

[a] SD: standard deviation of the average summary statistic (i.e. averaged mean values or the averaged median values, etc.).

compared with the control WBC (Dunnett's *C*-test, $P < 0.001$). The first dose (50 μg/mL) was already effective (*C*-test, $P < 0.001$). The presence of amifostine determined considerable protection against the genotoxic effectiveness of bleomycin: at 100 μg/mL, a significant effect was also evident (mean Comet length reduced to 19.29 vs. 26.58 μm, *C*-test $P < 0.001$). Furthermore, the first effective dose become 70 μg/mL (*C*-test, $P < 0.001$).

In acute myelogenous leukaemia cell line K562, genotoxicity increases with larger doses of bleomycin irrespective of amifostine pre-treatment (Table 3). The first dose with genotoxic effectiveness was 50 μg/mL both with (*C*-test, $P < 0.05$) and without (*C*-test, $P < 0.01$) amifostine, i.e. pre-treatment with amifostine did not protect K562 cells from bleomycin genotoxic action.

The median and 95th percentile values (Tables 2 and 3), confirmed the protective effectiveness of the pro-drug WR-2721 against DNA damage induced by bleomycin in WBC and its inefficacy in K562 cells. Another difference between WBC and K562 cells was the higher sensitivity against bleomycin in leukaemia cell line (*C*-test, $P < 0.01$).

The results in the three different experiments (Figs. 1 and 2) indicate reproducible findings on the genotoxic effects of bleomycin, both in WBC and K562, and the protective action of amifostine in WBC. The different behaviour of WBC and K562 cells in response to WR-2721 pre-treatment was evident.

A further series of experiments was performed to evaluate if individual sensitivity was maintained during the time or was modulated by exogenous agents (Table 5). Cells of the same donor were sampled about 4 months after the last sampling. An antimalaria prevention treatment (mefloquine) was finished 1 day before the sampling. Both bleomycin and amifostine effects are strongly depressed in sampling 2 with respect to sampling 1 (at 100 μg/mL bleomycin without amifostine, mean Comet length reduced to 19.17 vs. 27.65 μm, *C*-test $P < 0.001$; DNA damage measured as Comet length was reduced by amifostine from 19.17 to 18.70 μm in the second experiment vs. from 27.65 to 17.37 μm in the first, *C*-test $P < 0.001$). A recovery of bleomycin sensitivity was observed in the subsequent samplings. The amifostine

Table 3
DNA damage induced by different doses of bleomycin with (+) or without (−) amifostine pre-incubation in K562 cells[a]

Dose (μg/mL)	Amifostine	Average mean (μm ± SD)	Average median (μm ± SD)	Average 95th percentile (μm ± SD)	Dispersion ± SD (Variance/Mean)
		Comet length			
0	−	15.74 ± 0.21	14.43 ± 0.17	28.61 ± 0.50	2.93 ± 1.21
	+	16.12 ± 0.67	14.73 ± 1.42	28.74 ± 3.85	2.71 ± 1.67
50	−	25.71 ± 2.44	23.33 ± 4.40	43.18 ± 3.75	2.98 ± 2.56
	+	27.29 ± 2.58	25.17 ± 1.69	47.77 ± 1.80	3.33 ± 1.45
60	−	27.98 ± 2.88	26.43 ± 4.09	45.98 ± 5.68	3.40 ± 2.38
	+	28.35 ± 2.52	26.08 ± 2.52	48.28 ± 2.04	3.81 ± 1.46
70	−	31.66 ± 1.39	30.94 ± 3.53	51.68 ± 7.53	4.62 ± 2.40
	+	31.16 ± 3.14	29.71 ± 4.04	46.63 ± 4.01	3.49 ± 1.18
80	−	32.92 ± 0.64	29.03 ± 2.09	53.64 ± 1.72	4.05 ± 0.77
	+	32.46 ± 2.09	32.02 ± 1.67	47.11 ± 1.30	2.74 ± 0.49
90	−	35.40 ± 0.42	33.88 ± 1.08	58.25 ± 1.21	4.35 ± 1.16
	+	35.14 ± 3.05	34.92 ± 2.18	53.41 ± 2.87	3.23 ± 0.39
100	−	37.83 ± 1.04	36.90 ± 1.31	55.97 ± 0.85	3.34 ± 0.74
	+	37.71 ± 3.98	37.60 ± 3.70	52.57 ± 6.69	2.41 ± 0.60
		Tail moment			
0	−	12.01 ± 0.16	10.30 ± 0.12	23.88 ± 0.42	3.06 ± 1.14
	+	12.23 ± 0.51	10.70 ± 1.03	24.35 ± 3.26	2.50 ± 1.52
50	−	21.42 ± 2.03	18.57 ± 3.50	38.12 ± 3.31	3.10 ± 2.62
	+	23.26 ± 2.20	20.84 ± 1.40	43.58 ± 1.64	3.60 ± 1.19
60	−	23.39 ± 2.41	21.69 ± 3.36	41.04 ± 5.07	3.47 ± 2.54
	+	24.01 ± 2.13	21.40 ± 2.07	43.57 ± 1.84	3.78 ± 1.54
70	−	27.02 ± 1.19	26.02 ± 2.97	45.59 ± 6.64	4.33 ± 2.26
	+	26.31 ± 2.65	25.34 ± 3.45	43.69 ± 3.76	3.31 ± 0.80
80	−	28.49 ± 0.55	24.32 ± 1.75	51.51 ± 1.65	4.11 ± 0.42
	+	28.22 ± 1.82	27.82 ± 1.45	42.31 ± 1.17	2.54 ± 0.47
90	−	30.92 ± 0.37	29.50 ± 0.94	52.52 ± 1.09	4.40 ± 0.94
	+	30.52 ± 2.65	30.33 ± 1.89	48.74 ± 2.62	3.35 ± 0.04
100	−	32.80 ± 0.90	31.94 ± 1.13	50.99 ± 0.77	3.36 ± 0.82
	+	32.53 ± 3.43	32.84 ± 3.23	48.88 ± 6.22	2.58 ± 0.53

[a] SD: standard deviation of the average summary statistic (i.e. averaged mean values or the averaged median values, etc.).

cytoprotective effect was not recovered to the same extent. The relationship between antimalaria treatment and cell sensitivity is uncertain.

Table 4
Survival percentage (mean ± standard deviation) in WBC and K562 cells 24 hr after bleomycin treatment, with (+) or without (−) amifostine pre-treatment

Dose (μg/mL)	Amifostine	Cell survival (%)	
		WBC	K562
0	−	83.3 ± 5.1	79.5 ± 2.5
	+	95.8 ± 3.0	91.2 ± 5.1
50	−	90.2 ± 4.2	93.5 ± 3.3
	+	86.9 ± 2.8	92.7 ± 3.6
60	−	78.7 ± 3.3	91.7 ± 5.8
	+	84.1 ± 3.0	79.6 ± 3.1
70	−	87.1 ± 4.8	76.9 ± 3.9
	+	85.7 ± 3.1	90.7 ± 4.4
80	−	91.0 ± 4.7	92.9 ± 3.0
	+	90.1 ± 2.2	94.9 ± 2.3
90	−	84.3 ± 5.3	91.2 ± 5.1
	+	86.9 ± 4.4	81.8 ± 3.9
100	−	89.7 ± 5.2	92.9 ± 3.3
	+	86.1 ± 4.8	89.1 ± 3.3

4. Discussion

Recent advances in chemotherapy have focused on the benefit of high dose regimens, increasing the dose intensity of conventional chemotherapy. Dose intensity usually increases objective response rates of antineoplastic drugs and might, in some circumstances, improve the survival rate. Nevertheless, unacceptable cyto- and genotoxicity on normal cells often impair the proper management of patients, leading to dose reduction or treatment delay, thus reducing the efficacy, and potentially, the quality of life of patients. Therefore, considerable efforts have been made to manage, prevent, and delay treatment-related toxicity.

The phosphoaminothiol WR-1065, the active metabolite of the pro-drug amifostine, appears to selectively protect normal cells and tissues against cytotoxic exposure to radiation or chemotherapeutic agents.

Our previous study on the amifostine derivative thiol in WBC and a leukaemia cell line, K562, established a selective protection against melphalan-induced genotoxicity [22]. However, the ambiguous action of a thiol such as glutathione on bleomycin metabolism [11–14] and the

Table 5
Intra-individual variability of DNA damage induced by different doses of bleomycin in WBC of the same subject sampled at four different times (samplings 1–4)[a]

Dose (μg/mL)	Amifostine	Sampling 1 (07/15)	Sampling 2 (11/25)	Sampling 3 (12/02)	Sampling 4 (12/16)
		Comet length (μm)			
0	−	14.01 ± 2.71	13.54 ± 4.81	13.92 ± 3.08	14.88 ± 9.30
50	−	18.70 ± 11.09	14.85 ± 1.64	16.77 ± 9.29	21.27 ± 12.05
100	−	27.65 ± 11.98	19.17 ± 13.27	24.43 ± 12.84	29.25 ± 12.12
0	+	14.08 ± 6.72	13.97 ± 5.99	13.65 ± 4.26	14.92 ± 5.99
50	+	15.83 ± 10.55	14.87 ± 2.42	15.01 ± 2.22	19.31 ± 12.53
100	+	17.37 ± 6.51	18.70 ± 14.24	19.29 ± 11.55	27.96 ± 10.33
		Tail moment (μm)			
0	−	10.64 ± 2.06	10.10 ± 3.59	9.63 ± 2.13	10.91 ± 6.82
50	−	14.81 ± 8.78	10.86 ± 1.20	12.69 ± 7.03	17.38 ± 9.85
100	−	23.14 ± 10.03	14.40 ± 9.97	19.87 ± 10.44	25.09 ± 10.40
0	+	10.29 ± 4.91	9.61 ± 4.12	9.10 ± 2.84	10.59 ± 4.25
50	+	11.48 ± 7.65	10.27 ± 1.67	11.11 ± 1.64	15.21 ± 9.87
100	+	13.60 ± 5.10	13.99 ± 10.65	15.17 ± 9.08	23.93 ± 8.84

[a] Mean ± standard deviation.

disagreement in findings on amifostine protection against bleomycin-induced severe side effects [15–17] suggested the need for a study to be carried out on amifostine effectiveness against bleomycin-induced genotoxicity in normal and tumour cells.

WR-2721 appears to selectively protect healthy leukocytes but not K562 tumoral cells. This action is mainly based on the efficient dephosphorylation of aminothiol by alkaline phosphatase in WBC, whereas K562 cells are unable to activate amifostine.

Data on the inter- and intra-individual sensitivity to bleomycin-induced genotoxicity suggest that individual metabolic/genetic differences, such as drug metabolising enzyme polymorphisms, and other factors relating to lifestyle may be responsible for response variability and should be considered during clinical chemotherapy treatment. Amifostine protective action also appears to be modulated by exogenous (and endogenous?) factors when tested in the same subject. Contrasting results on amifostine protection against severe bleomycin-induced side effects in different mammalian species might also be clarified by our data.

The findings indicate the suitability of this technique as a primary screening method for *in vitro* and *in vivo* studies on drug–DNA interactions and their modulations by endogenous/exogenous factors. Furthermore, the data suggest a possible application of the Comet assay in appropriate clinical settings to test the sensitivity of patients when undergoing chemotherapy.

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